Amino acid substitution K186E in the canine influenza virus H3N8 NS1 protein restores its ability to inhibit host gene expression

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ABSTRACT

Canine influenza viruses (CIVs) are the causative agents of canine influenza, a contagious respiratory disease in dogs, and include the equine-origin H3N8 and the avian-origin H3N2. Influenza A virus (IAV) non-structural protein 1 (NS1) is a virulence factor essential for counteracting the innate immune response. Here, we evaluated the ability of H3N8 CIV NS1 to inhibit host innate immune responses. We found that H3N8 CIV NS1 was able to efficiently counteract interferon (IFN) responses but was unable to block general gene expression in human or canine cells. Such ability was restored by a single amino acid substitution in position 186 (K186E) that resulted in NS1 binding to the 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30), a cellular protein involved in pre-mRNA processing. We also examined the frequency distribution of K186 and E186 among H3N8 CIVs and equine influenza viruses (EIVs), the ancestors of H3N8 CIV, and experimentally determined the impact of amino acid 186 in the ability of different CIV and EIV NS1s to inhibit general gene expression. In all cases, the presence of E186 was responsible for the control of host gene expression. Contrastingly, the NS1 protein of H3N2 CIV harbors E186 and blocks general gene expression in canine cells. Altogether, our results confirm previous studies on the strain-dependent ability of NS1 to block general gene expression. Moreover, the observed polymorphism on amino acid 186 between H3N8 and H3N2 CIVs might be the result of adaptive changes acquired during long-term circulation of avian-origin IAVs in mammals.

IMPORTANCE

Canine influenza is a respiratory disease of dogs caused by two CIV subtypes, the H3N8 and H3N2 viruses of equine and avian origin, respectively. Influenza NS1 is the main viral factor responsible for the control of host innate immune responses and changes in NS1 can play an important role in host adaptation. Here we assessed the ability of H3N8 CIV NS1 to inhibit host innate immune responses and gene expression. The H3N8 CIV NS1 did not block host gene expression but this activity was restored by a single amino acid substitution (K186E), which was responsible for NS1 binding to the host factor CPSF30. In contrast, the H3N2 CIV NS1, that contains E186, blocks general gene expression. Our results suggest that the ability to block host gene expression is not required for influenza replication in mammals but might be important in the long-term adaptation of avian-origin influenza viruses to mammals.

INTRODUCTION

Influenza A viruses (IAVs) are single-stranded, negative-sense, segmented RNA viruses that belong to the *Orthomyxoviridae* family (1, 2). They are classified on the basis of the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (1, 2). While IAVs mostly circulate in wild aquatic birds (3-6), some have become established as novel lineages in various avian and mammalian hosts (7, 8). In addition, many IAVs cause sporadic spillover infections in humans and other mammals (9, 8, 4).

Two canine influenza viruses (CIVs) have emerged in the last decade. The first one is of the H3N8 subtype, and was first reported during an outbreak of respiratory disease in Florida, USA, in 2004 (10-12). H3N8 CIV originated from equine influenza virus (EIV), an avian-origin IAV that has been circulating in horses since the 1960s (10, 13). Another recently emerged CIV is the avian-origin H3N2 subtype, originated in China, which has been circulating in Asia since ~2006 (14) and was introduced to North America in 2015 (15). Since CIVs are the result of successful cross-species virus transfers, it is of interest to understand how they have succeeded in their new host, and whether they constitute potential threats to the global canine population (16, 7, 8) and perhaps to other species, including humans. Understanding the determinants of pathogenesis and host range of CIVs is essential to shed light on the mechanisms of disease in dogs and to provide insights into the general mechanisms of IAV host range in other species.

The host innate immune responses constitute an essential part of the host defenses that restrict viral infections. These are mainly mediated by the induction of an interferon (IFN) response that results in the expression of a broad range of IFN-stimulated genes (ISGs), some of which possess antiviral activity (17). IAVs encode the non-structural

protein 1 (NS1), a multifunctional protein that is a molecular determinant of IAV virulence and is the main viral antagonist of the IFN response (18-20). NS1 acts at multiple levels of the IFN signaling pathway: it has been shown to impair the expression of ISGs (18) and to directly inhibit specific ISGs, such as protein kinase R (PKR) (18) and 2'-5' oligoadenylate synthetase and ribonuclease L (RNase L) (21). NS1 can also inhibit the production of IFN at the pre-transcriptional level by sequestering double-stranded (ds)RNA and subsequently decreasing the activation of retinoic acid inducible gene 1 (RIG-I) (22-24), or by inhibiting the tripartite motif family (TRIM)-25-mediated RIG-I ubiquitination (22, 24). NS1 can also impair the IFN response by blocking general host gene expression by binding the 30-kDa subunit of the cleavage and polyadenylation specificity factor 30 (CPSF30). The ability of NS1 to bind CPSF30 and block host gene expression is not conserved in all IAVs. Indeed, it has been shown that naturally occurring IAVs that infect humans, such as the swine-origin 2009 pandemic H1N1 (pH1N1) virus (25), the avian-origin H7N9 (26) and H5N1 (27) viruses, as well as the laboratory strain A/Puerto Rico/8/34 (PR8) (28), do not bind CPSF30. The amino acid(s) involved in the interaction with CPSF30 have been mapped to different NS1 residues (26, 29, 30, 25, 28, 31-34).

Here we examined the ability of H3N8 CIV NS1 to counteract the IFN response, and found that this protein blocks the induction of IFN and ISGs without blocking general host gene expression. However, a single amino acid substitution (K186E) results in the recovery of the ability to block general host transcription. These results confirm the notion that the specific functions of NS1 protein are strain-dependent and likely vary with the level of virus adaptation to the host.

MATERIALS AND METHODS

Cell lines

Human embryonic kidney 293T, HEK293T (American Type Culture Collection, ATCC CRL-11268); Madin-Darby canine kidney, MDCK (ATCC CCL-34); and BSR-T7 (kindly provided by Dr. Karl-Klaus Conzelmann) (35) cells were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.), 10% fetal bovine serum (FBS), and 1% PSG (penicillin, 100 units/ml; streptomycin 100 µg/ml; L-glutamine, 2 mM) (36, 37). A72 cells (ATCC CRL-1542) were grown in 50/50 McCoy's 5A (Gibco)/Leibovitz's L-15 (Gibco) with 5% FBS and 1% PSG.

Virus rescue

Virus rescues were performed as previously described (38, 37). Briefly, co-cultures (1:1) of HEK293T/MDCK cells (6-well plate format, 10^6 cells/well, triplicates) were co-transfected in suspension, using Lipofectamine 2000 (LPF2000; Invitrogen), with 1 µg each of the seven A/canine/NY/dog23/2009 H3N8 ambisense wild-type (WT) plasmids (pDZ-PB2, -PB1, -PA, -NP, -NA, -M, -HA) (13) plus the ambisense WT (pDZ-NS) or mutant K186E (pDZ-NS K186E) plasmids. At 12 hours post-transfection (hpt), the medium was replaced with DMEM containing 0.3% bovine serum albumin (BSA), 1% PSG, and 1 µg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). At 48 hpt, tissue culture supernatants (TCS) were used to infect fresh monolayers of MDCK cells (6-well plate format, 10^6 cells/well, triplicates). At 3 days post-infection (pi), recombinant viruses were plaque purified and scaled up in MDCK cells. Stocks were titrated by plaque assay on MDCK cells (38, 37), and the identity of the NS segment was confirmed by restriction analysis and sequencing.

Virus growth kinetics and immunostaining

To evaluate viral growth kinetics, MDCK cells or A72 cell (12-well plate format, $5x10^5$ cells/well, triplicates) were infected at low (0.001) and high (3) multiplicity of infection (MOI) and the TCS were collected at the indicated time points. Viral titers were determined by immunofocus assay (fluorescent forming units [FFU]) in MDCK cells as previously described (38, 37). The mean value and standard deviation were calculated using Microsoft Excel software. For the immunostaining, confluent MDCK cell monolayers (6-well plate format, 10^6 cells/well) were infected with 10-fold serial dilutions of H3N8 CIV WT or K186E viruses. After infection, monolayers were overlaid with agar and incubated for 72 h at 33ºC. Then, cells were fixed with 4% paraformaldehyde (PFA), and the overlays were removed. Fixed cells were then permeabilized (0.5% Triton X-100 in PBS for 15 min at room temperature) and used for immunostaining as previously described (38, 37) using anti-NP MAb (ATCC, HB-65) (American Type Culture Collection, ATCC) and vector kits (Vectastain ABC kit and DAB HRP Substrate Kit: Vector), according to manufacturer's specifications.

RT-PCRs

Total RNA from CIV or EIV-infected (MOI 3) MDCK cells (6-well plate format, 10^6 cells/well) was collected at 18 hpi. and purified using TRIzol (Invitrogen) according to the manufacturer's specifications. cDNA synthesis for NS viral (v)RNAs or NS1 mRNAs were performed using SuperScript® II Reverse Transcriptase (Invitrogen) and specific primers. cDNAs were used as templates for semi-quantitative PCR with primers specific for the NS vRNA (A/canine/NY/dog23/2009 H3N8) or NS1 mRNAs (A/canine/Florida/2004 H3N8, A/equine/Pennsylvania/1/2007 H3N8,

A/equine/Miami/1963 H3N8 and A/canine/IL/41915/2015 H3N2). Primer sequences are available upon request. Influenza A/equine/Pennsylvania/1/2007 and A/equine/Miami/1963 H3N8 were obtained from BEI Resources (NR-13426 and NR-3175, respectively). Influenza A/canine/Florida/2004 H3N8 and influenza A/canine/IL/41915/2015 H3N2 were provided by the Baker Institute for Animal Health at Cornell University.

For quantitative RT-PCRs (qRT-PCRs), MDCK cells (12-well plate format, $5x10^5$ cells/well, triplicates) were mock infected or infected (MOI of 3) with NS1 WT and K186E H3N8 CIVs. At 12 or 24 hpi, total RNA was extracted using an RNeasy minikit (Qiagen) following the manufacturer's recommendations. Reverse transcription reactions were performed at 37°C for 2 h using the high-capacity cDNA transcription kit and an oligodT primer to amplify mRNAs. Quantitative PCRs were performed using TaqMan IFNβ and IFN-induced protein with tetratricopeptide repeats 2 (IFIT2) assays (Applied Biosystems) specific for the *Canis familiaris* (dog) genes (Cf03644503_s1 and Cf02645026_m1, respectively). Quantification was achieved using the $2^{-\Delta\Delta CT}$ method (39).

Plasmids

To engineer polymerase II expression pCAGGS plasmids (40) containing the NS1 sequences fused to an HA epitope tag (YPYDVPDYA) at the N terminus (pCAGGS-HA-NH2) (23) we used standard molecular biology techniques. The different NS1 genes were amplified by RT-PCR using oligonucleotides with the appropriate flanking restriction sites (SmaI and XhoI) for cloning into pCAGGS-HA-NH2. NS1 open reading frames were also cloned in a pGEM-T plasmid (Promega) to introduce the different

mutations by site-directed mutagenesis using specific primers. To abolish NS mRNA splicing, two silent mutations were introduced at nucleotides (nt) 501 and 504 of NS1 (30, 33). NS1 mutants were subcloned from the pGEM-T to the pCAGGS-HA-NH2 using SmaI and XhoI. Plasmids encoding NS1 variants under the control of the phage T7 polymerase were obtained by subcloning NS1 from the pCAGGS-HA-NH2 plasmids into pcDNA3 plasmid using EcoRI and XbaI restriction enzymes. pCAGGS and pDNA3 plasmids encoding the NS1 proteins of influenza A/Puerto Rico/8/34 (PR8) and influenza A/Brevig Mission/01/1918 (BM/18) H1N1 viruses were previously described (28, 23). To generate a H3N8 CIV containing the mutation K186E, the mutated NS viral segment was cloned into the ambisense pDZ plasmid for virus rescue. All the plasmid constructs were confirmed by sequencing (ACGT, Inc.). Primers used for the construction of the different plasmids are available under request.

Inhibition of host gene expression

To evaluate the effect of viral NS1 proteins on host protein synthesis, HEK293T or MDCK cells (12-well plate format, $3x10^5$ cells/well, triplicates) were transiently co-transfected, using LPF2000, with 2 µg/well of pCAGGS-HA NH2 NS1 protein expression plasmids, or an empty plasmid as a control, together with 50 ng/well of pCAGGS plasmids expressing the green fluorescent protein, GFP (28) and Gaussia luciferase**,** Gluc (41). For T7-driven expression, BSR-T7 cells (12-well plate format, $3x10^5$ cells/well, triplicates) were transiently co-transfected, as indicated above, with 2 µg/well of pCAGGS-HA NH2 NS1 protein expression plasmids, or an empty plasmid as a control, together with 0.5 µg/well of the reporter expression plasmids pCITE-FFLuc and pCITE-GFP, which express Firefly luciferase (FFluc) or GFP under the control of

the T7 promoter (38). At 24 hpt , cells were analyzed for GFP expression under a fluorescent microscope and Gluc (HEK293T and MDCK cells) or FFluc (BSR-T7 cells) activities were quantified from TCS (Gluc) or cell lysates (FFluc) using a Biolux Gaussia luciferase reagent (New England Bio-Labs) or a FFluc luciferase reporter buffer (Promega) and a Lumicount luminometer. The mean values and standard deviations were calculated using Microsoft Excel software.

Inhibition of IFN-β and ISRE promoters

The assays to evaluate the effect of NS1 proteins on the inhibition of IFN-β and IFN stimulated response element (ISRE) promoters have been previously described (28). Briefly, HEK293T cells (12-well plate format, $3x10^5$ cells/well, triplicates) were co-transfected with 1 µg/well of the pCAGGS-HA NH2 NS1-expressing plasmids, or empty plasmid as control, together with 0.5 µg/well of plasmids expressing FFluc under the control of the IFN-β or the ISRE promoters (pIFN-β -FFluc and pISRE-FFluc, respectively) (28), and 0.5 µg/well of plasmids expressing GFP or RFP fused to the chloramphenicol acetyltransferase (CAT) driven by the IFN-β or the ISRE promoter (pIFNβ-GFP/CAT and pISRE-RFP/CAT, respectively) using a calcium phosphate-based mammalian transfection kit (Stratagene). At 18 hpt, cells were infected (MOI 3) with Sendai virus (SeV), Cantell strain (28), and at 18 hpi cells were analyzed for IFN-β or ISRE promoter activation by GFP (IFN-β) or RFP (ISRE) expression under a fluorescent microscope. FFluc activity was quantified from cell lysates using a FFluc luciferase reporter buffer (Promega) and a Lumicount luminometer (PacKard). Reporter gene activation is shown as relative light units (RLU) compared to SeV-infected cells in the

absence of NS1 protein (empty plasmid). The mean values and standard deviations were calculated using Microsoft Excel software.

Protein gel electrophoresis and Western blot analysis

Total proteins from cells lysates were separated using 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% dried skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (T-PBS) and incubated overnight at 4°C with anti-HA (for NS1; Sigma) or anti-FLAG (for F2/F3 CPSF30; Sigma) polyclonal antibodies (pAb). A monoclonal antibody (mAb) specific for actin (Sigma) was used as an internal loading control. Bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated antibodies (GE Healthcare) against immunoglobulins of different species (mouse or rabbit). Proteins were detected by chemiluminescence using the SuperSignal West Femto maximum-sensitivity substrate (Thermo Scientific) following the manufacturer's recommendations and photographed using a Kodak Image Station.

NS1 interaction with CPSF30

HA-tagged NS1 proteins were synthesized *in vitro* using pcDNA3 plasmids and the TNT7 transcription/translation kit (Promega) following the manufacturer's recommendations. HEK293T cells (6-well format, 1.5 $\times 10^6$ cells/well) were transiently transfected with 2 µg/well of a pCAGGS plasmid expressing a FLAG-tagged version of the F2/F3 region of the human CPSF30 (FLAG-F2/F3 CPSF30) (34). At 48 hpt, cells were lysed in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 5% glycerol, and 1% Triton X-100 supplemented with a complete mini protease inhibitor cocktail (Pierce). Cleared cell lysates were incubated overnight at 4°C with the *in vitro*-synthesized NS1

proteins and 20 µl of an anti-FLAG affinity resin (Sigma). After extensive washing, precipitated proteins were dissociated from the resin using Laemmli buffer and analyzed by Western blotting as described above.

RESULTS

H3N8 CIV NY09 NS1 protein does not block host gene expression in human or canine cells

To evaluate whether H3N8 CIV NS1 protein could block host gene expression, human HEK293T or canine MDCK cells were co-transfected with plasmids encoding GFP or GLuc together with individual plasmids encoding the NS1 genes of either A/canine/NY/dog23/2009 H3N8 (CIV NY09), A/Puerto Rico/8/34 H1N1 (PR8) and A/Brevig Mission/1/18 H1N1 (BM/18) (**Fig. 1**). Cells transfected with empty plasmid were also used as an internal control. We have previously described that PR8 NS1 is not able to block host gene expression. However such ability can be restored by introducing amino acid substitutions S103F and I106M in PR8 NS1 (28). Contrary, the NS1 protein from BM/18 is a strong inhibitor of host gene expression (42, 28). At 24 hpt, GFP expression was evaluated using fluorescence microscopy (**Figs. 1A and 1D**) and Gluc expression levels were quantified in a luminometer (**Figs. 1B and 1E**). As expected, PR8 NS1 protein did not block expression of GFP and/or Gluc whereas BM/18 NS1 inhibited protein expression of both reporter genes (28). CIV NY09 NS1 protein did not inhibit either GFP or Gluc expression. The NS1 proteins from PR8 or CIV NY09 were detected by Western blot (**Figs. 1C and 1F**) but this was not the case for the NS1 protein of BM/18, because this NS1 is inhibiting its own synthesis (28). Our results obtained with both human (**Figs. 1A-1C**) and canine (**Figs. 1D-1F**) cells were similar, suggesting that the inability of CIV NY09 NS1 protein to block host gene expression does not depend on the origin of the cells.

A single substitution (K186E) in the NS1 protein of H3N8 CIV NY09 restores its ability to block host gene expression

To identify the amino acid(s) in H3N8 CIV NY09 NS1 protein responsible for the lack of inhibition of host gene expression we aligned the NS1 protein sequences of the viruses used in our reporter-based assay (**Fig. 1**) and also included two additional viruses: A/Texas/36/91 H1N1 (TX/91, which binds CPSF30), and the pandemic A/California/04/09 H1N1 (pH1N1, which does not bind CPSF30) (**Fig. 2A**). We identified eight amino acids (P77, D96, T112, E139, F185, K186, K193 and I194) in the H3N8 CIV NY09 NS1 protein that could have a potential role in the lack of inhibition of host gene expression (**Fig. 2A**, gray). To test the contribution of these residues to the inhibition of host gene expression, we introduced mutations P77L, D96E, T112A, E139D, F185L, K186E, K193R and I194V into the CIV NY09 NS1 protein and tested their ability to block general gene expression in our reporter-based assay in MDCK cells (**Figs. 2B-2C**). A single substitution (K186E) introduced the ability of H3N8 CIV NY09 NS1 protein to inhibit general host gene expression as measured by GFP (**Fig. 2B**) and Gluc (**Fig. 2C**) expression levels. Moreover, the K186E mutant construct was the only one that could not be detected by Western blot, suggesting it was inhibiting its own expression (**Fig. 2D**).

We next evaluated if the ability of H3N8 CIV NY09 NS1 K186E protein to inhibit host gene expression was dose-dependent (**Figs. 3A-3B**). MDCK cells were co-transfected with the pCAGGS plasmid expressing Gluc together with different amounts of the plasmids encoding CIV NY09 NS1 WT or the K186E mutant. Clearly, CIV NY09 NS1 K186E protein showed a dose-dependent inhibitory effect on Gluc expression **(Fig. 3A)** and inhibition of its own expression (**Fig. 3B**). H3N8 CIV NY09 NS1 WT protein did not have an effect on reporter gene expression **(Fig. 3A)** and was detected by Western blot

only when 2, 1 or 0.5 µg of plasmid was transfected, most likely because of the limit of detection of the assay (**Fig. 3B**).

To test if CIV NY09 NS1 K186E protein inhibition of host gene expression was due to inhibiting transcripts produced by the cellular RNA polymerase II, we used a variation of the assay where reporter gene expression (GFP and FFluc) was under the control of a bacteriophage T7 polymerase promoter (38). BSR-T7 cells constitutively expressing T7 RNA polymerase (35) were co-transfected with reporter GFP and FFluc plasmids under the control of the T7 polymerase, together with plasmids encoding the H3N8 CIV NY09 NS1 WT or K186E, the NS1 proteins from PR8 or BM/18 or empty plasmid as internal controls. At 24 hpt, GFP and FFluc expressions were analyzed using fluorescence microscopy (**Fig. 3C**) or a luminometer (**Fig. 3D**), respectively. In this assay, none of the NS1 constructs showed any effect on reporter gene expression, demonstrating that only nuclear transcripts produced by cellular RNA polymerase II were targets of the inhibitory effect of NS1 WT or mutant constructs. As expected, when NS1 protein expression was analyzed by Western blot, H3N8 CIV NY09 K186E and BM/18 NS1 proteins were not detected (**Fig. 3E**).

K186E substitution introduces the ability of H3N8 CIV NY09 NS1 protein to bind to CPSF30

It has been shown that NS1 proteins of some IAV strains interact with CPSF30, blocking host gene expression (26, 29, 30, 25, 28, 31-34). The interaction of NS1 and CPSF30 is mediated by the effector domain of the former and the F2/F3 region of the latter (26, 29, 30, 25, 28, 31-34). Importantly, the F2/F3 region of CPSF30 is conserved between species including the ones relevant for this study (human, dogs and horses)

(data not shown). Given the ability of the H3N8 CIV NY09 NS1 K186E protein to inhibit host gene expression, we hypothesized that this phenotype was due to a restored ability to bind CPSF30. We evaluated the interaction between H3N8 CIV NY09 NS1 WT or K186E proteins and CPSF30 by co-immunoprecipitation (**Fig. 4A**). Cell extracts from human HEK293T cells transfected with a plasmid encoding an N-terminal FLAG-tagged F2/F3 region of CPSF30 (34) were incubated with *in vitro* transcribed and translated H3N8 CIV NY09 NS1 WT and K186E proteins or with PR8 NS1 protein as control, and agarose beads conjugated with an anti-FLAG antibody. As expected, PR8 NS1 and H3N8 CIV NY09 NS1 WT proteins did not co-immunoprecipitate with CPSF30 (28), however, CIV NY09 NS1 K186E protein did (**Fig. 4A**). The three dimensional structure of influenza A/Udorn/72 H3N2 NS1 bound to the F2/F3 domain of CPSF30, shows that NS1 residue 186 is closely located to the CPSF30 protein (**Fig. 4B**), providing a likely explanation for the effect of this amino acid change in the binding to CPSF30 (29). Altogether, our results indicate that the K186E substitution restores the ability of H3N8 CIV NY09 NS1 protein to bind CPSF30.

Effect of H3N8 CIV NY09 NS1 protein on inhibition of IFN responses

To analyze the effect of H3N8 CIV NY09 NS1 WT and K186E proteins to counteract IFN responses, human HEK293T cells were co-transfected with pCAGGS plasmids expressing PR8, BM/18, CIV NY09 WT and K186E NS1 proteins, together with plasmids expressing FFluc and GFP under the control of the IFN-β promoter (**Figs. 5A-5C**) or FFluc and RFP under the control of an ISRE promoter (**Figs. 5D-5F**). At 18 hpt, cells were mock-infected or infected with the Cantell strain of SeV for 18 h to induce activation of both promoters. Then, IFN-β and ISRE promoter activation was

364 determined by measuring expression levels of GFP or RFP (**Figs. 5A and 5D**) and
365 FFluc (**Figs. 5B and 5E**). As expected, SeV infection induced robust activation of the
366 respective promoters (IFN-β, **Figs. 5A-5B;** and ISRE, **Figs. 5D-5E**) in cells transfected
367 with empty plasmid. On the other hand, and consistent with previous results (18), SeV-
368 infected cells transfected with the different NS1-expressing plasmids showed less
369 activation of both promoters compared with those transfected with empty plasmid.
370 Moreover, when NS1 protein expression levels were evaluated by Western blot, only
371 PR8 and H3N8 CIV NY09 WT NS1 proteins were detected (**Figs. 5C and 5F**). These
372 results indicate that K186E substitution did not affect the ability of H3N8 CIV NY09 NS1
373 protein to inhibit SeV-mediated activation of IFN-β or ISRE promoters. In addition, these
374 results suggest that H3N8 CIV NY09 NS1 protein has IFN antagonistic properties
375 despite not being able to block general host gene expression in either human or canine
376 cells (**Fig. 1**).

377 **Generation and characterization of WT and K186E H3N8 NY09 CIVs**

378 To analyze whether the H3N8 CIV NY09 NS1 K186E mutation had an effect on virus
379 replication, we generated recombinant H3N8 NY09 CIVs containing the WT or the
380 K186E substitution in NS1 (13). We first confirmed the identity of the recombinant
381 viruses by RT-PCR and restriction analysis of the NS segment. K186E substitution
382 removed a SwaI restriction site in NS1, which was used as a genetic marker to
383 distinguish H3N8 NY09 WT and K186E recombinant CIVs (**Fig. 6A**). In addition, the NS
384 viral (v)RNA was sequenced to ensure the absence of additional mutations (data not
385 shown). The amino acid substitution K186E in H3N8 NY09 NS1 protein did not affect
386 the amino acid sequence of the viral NEP, which is produced using an alternative

splicing mechanism from the same NS vRNA (18, 19). Next, we compared both viruses' ability to spread between neighboring cells by plaque assay in MDCK cells (**Fig. 6B**), as well as their multicycle and single cycle growth kinetics in MDCK (**Figs. 6C and 6D**, respectively) and in A72 (**Figs. 6E and 6F,** respectively) canine cells. Canine A72 cells are a well-established tissue culture system to study viruses infecting dogs, including parvovirus and CIV (13, 43). CIV NY09 NS1 K186E displayed a similar plaque phenotype in MDCK cells as the parental virus (**Fig. 6B**). Interestingly, multicycle replication experiments using MDCK (**Fig. 6C**) or A72 (**Fig. 6E**) cells infected at low MOI (0.001) revealed that the recombinant H3N8 NY09 WT and K186E CIVs grew with similar kinetics. However, when cells were infected at high MOI (3), the WT virus grew at lower titers than the K186E mutant in both cell lines (**Figs. 6D and 6F**). These data indicate that the defect in efficient CPSF30 binding and inhibition of host protein expression by CIV NY09 NS1 protein only slightly affects virus replication *in vitro*, as previously described for other IAVs (30, 18, 25, 28, 33).

To further analyze the ability of the recombinant NY09 H3N8 NS1 WT and K186E mutant CIVs to inhibit ISG IFIT2 (**Fig. 7A and 7B**) or IFNβ (**Fig. 7C and 7D**) responses, MDCK cells were infected (MOI 3) and at 12 (**Fig. 7A and 7C**) or 24 (**Fig. 7A and 7C**) hpi, expression of IFIT2 and IFNβ were measured at the mRNA level by qRT-PCR. Interestingly, the levels of IFIT2 expression were higher in NY09 H3N8 NS1 WT-infected MDCK cells than in mutant K186E-infected cells at both times pi (**Fig. 7A and 7B**). These data indicate that the amino acid change K186E increase the ability of NS1 to inhibit ISGs during viral infection. On the other hand, both viruses were able to inhibit the expression of IFNβ at similar levels (**Fig. 7C and 7D**).

The ability of H3N8 CIV NS1 protein to block host gene expression was lost during virus evolution

We next investigated if the inability to block host gene expression by H3N8 CIV NS1 protein was conserved along its evolution by comparing the NS1 amino acid sequences of H3N8 viruses phylogenetically related to CIV NY09, including A/canine/Florida/2004 H3N8 (CIV FL04) and the equine influenza viruses (EIVs) A/equine/Pennsylvania/1/2007 H3N8 (EIV Pen07) and the early A/equine/Miami/1963 H3N8 (EIV Mi63) (**Fig. 8)**. The K186 was mostly conserved as EIV Mi63 is the only virus that displays E186 (**Fig. 8A and Fig. 9**). We evaluated the ability of the aforementioned NS1 proteins to block host gene expression in MDCK cells, as previously described (**Fig. 1**). As expected, only the NS1 protein of EIV Mi63 blocked expression of GFP and/or Gluc (**Figs. 8B and C**). Similar results were obtained when NS1 protein expression levels were evaluated by Western blot (**Fig. 8D**). We confirmed that amino acid substitution E186K was responsible for the lack of inhibition of host gene expression when we introduced E186 in CIV FL04 and EIV Pen07 NS1 proteins and K186 in EIV Mi63 NS1 (**Figs. 8B-8D**).

EIV Mi63 was the first EIV isolated after the virus was transmitted from an avian host to horses, suggesting that the ability to inhibit host gene expression was lost during the evolution of EIV in horses but before the virus was transmitted to dogs in 2004 (**Fig. 9**). To assess the timeline describing the period over which amino acid substitution E186K was incorporated into NS1, the percent of H3N8 EIVs and CIVs strains whose NS1 sequence encoded the different amino acids were plotted (**Fig. 9**). Notably, database

analysis suggests that amino acid substitution E186K occurred early in the 70s in EIVs and has remained stable since then (**Fig. 9**). Moreover, all H3N8 CIV strains analyzed encode amino acid K186, indicating that since EIV transferred into dogs in 2004, K186 has been maintained (**Fig. 9**).

The avian-origin H3N2 CIV NS1 protein is able to block host gene expression

To determine if the inability to block general gene expression was a feature of influenza viruses of dogs, we next examined the NS1 sequences of H3N2 CIVs as this lineage has a different origin from H3N8 CIV. Interestingly, all the H3N2 CIV NS1 proteins exhibit E186 (data not shown), suggesting that the NS1 proteins of this lineage would block host gene expression. To confirm this, we co-transfected MDCK cells with the reporter expressing plasmids (**Fig. 1**) and the H3N2 CIV NS1 protein efficiently inhibited the expression of GFP and Gluc (**Figs. 10A and 10B,** respectively). Moreover, Western blot analysis further confirmed the ability of H3N2 CIV NS1 protein to inhibit host gene expression, including its own expression (**Fig. 10C**). Notably, this phenotype was reverted when the amino acid substitution E186K was introduced into the H3N2 CIV NS1 protein (**Fig. 10**). These data suggests that the ability to block general host gene expression is strain-dependent, similar to what has been observed with human influenza viruses.

DISCUSSION

The continued interspecies transmission of IAVs to humans and other mammals is a constant threat (44-46, 4). As dogs are popular companion animals and can support the replication of multiple IAV subtypes such as H3N8, H3N1, H3N2, H5N1, H5N2 and H1N1 (47, 48, 10, 49-52, 11, 53-58), they could act as intermediate hosts for IAV reassortment or human exposure (7). Various human IAVs such as PR8, A/Udorn/307/72 H3N2 and pH1N1 replicate in the respiratory tract of the dog and viable reassortant viruses between H3N8 CIV and pH1N1 IAV have been reported (59). In addition, it has been shown that dogs can be co-infected by pH1N1 and H3N2 CIV, leading to the generation of viable reassortants (60, 53). This highlights the need for monitoring the evolution of CIVs and studying the mechanism of IAV adaptation to new hosts.

IAVs possess mechanisms to antagonize host IFN and IFN-induced responses (61, 62). IAV NS1 is a multifunctional viral protein and the main factor to counteract host innate immune responses, allowing the virus to efficiently replicate during infection (63, 18). One mechanism of inhibition of innate immune responses is mediated through the binding of IAV NS1 to CPSF30, which blocks the processing of pre-mRNAs in the nucleus and suppresses the expression of host genes, including IFN and ISGs (26, 29, 30, 25, 28, 31-34). However, CPSF30-binding is not essential for successful IAV infection since some NS1 proteins do not bind CPSF30 (26, 29, 30, 25, 28, 31-34). The reasons for such variation are not well understood. However, it could be possible that in mammals, the lack of inhibition of important host genes (e.g. cytokines) prevents

478 infection by other pathogens that could interfere and/or compete with viral replication
479 and dissemination.

480 Our results show that H3N8 CIV NS1 protein inhibits the induction of IFN and ISGs
481 (**Fig. 5**), but does not bind CPSF30 (**Fig. 4**) and therefore is unable to block host gene
482 expression in either canine or human cells (**Fig. 1**). NS1 residue 186 proved to be a
483 main determinant in blocking general gene expression (**Fig. 2**), and the K186E
484 substitution introduced both the NS1-CPSF30 interaction (**Fig. 4**) and gene expression
485 inhibition (**Fig. 2**). This inhibition of host gene expression was dose dependent and
486 specific for polymerase II nuclear expressed transcripts (**Fig. 3**).

487 Our results are consistent with previous work using A/Udorn/72 H3N2 NS1 protein,
488 where residues around position 186 (amino acids 184 to 188; GLEWN) are important for
489 binding to CPSF30 and inhibiting host gene expression (32), and were important for
490 efficient viral replication (32). Interestingly, we found that CIV NY09 K186E replicated
491 better than the WT virus in two canine cell lines, but only at high MOI (**Fig. 6**).
492 Moreover, while both viruses were able to inhibit induction of IFNβ to a similar extend,
493 CIV NY09 mutant K186E inhibited the expression of ISG more efficiently than WT virus
494 (**Fig. 7**). However, the different abilities of CIV NY09 NS1 WT and K186E proteins to
495 block host gene expression could have a more significant role in the natural host, the
496 dog.

497 Our study also confirms that NS1 binding to CPSF30 and the resulting block in
498 general gene expression does not impair the ability of this NS1 to counteract IFN
499 induction (**Fig. 5**). As indicated for other IAVs (27, 30, 18, 25, 33, 34) our results clearly
500 show that the NS1 proteins of different IAV strains that infect the same host can differ in

501 their strategy to overcome the innate immune response. We showed that the avian-
502 origin H3N2 CIV NS1 protein -which contains E186- inhibits host gene expression (**Fig.**
503 **10**) and that the same is observed with A/equine/Miami/63, an early EIV (the ancestor of
504 H3N8 CIV) that possess E186 (**Fig. 8**). As both EIV H3N8 and H3N2 CIV are avian-
505 origin viruses, E186 might be important during the initial stages of IAV adaptation in
506 mammals (Chauche, Nogales et al., submitted) and that K186 provides increased
507 fitness at later evolutionary stages. Notably, since introduced in EIV in the early 70s,
508 amino acid substitution E186K has remained stable in both EIV and CIV strains (**Fig. 9**).

509 It has been recently reported that the PA-X protein from both H3N8 EIVs and CIVs
510 are able to suppress host gene expression (64). It is possible that EIV and CIV H3N8
511 NS1 proteins lost the ability to inhibit gene expression because of the ability of the viral
512 PA-X to suppress host protein expression (64). In fact, we have recently described that
513 inhibition of host protein expression by influenza virus is subject to a strict balance,
514 which can determine the successful progression of viral infection (65). Thus, it is
515 possible that H3N8 EIV and CIV NS1 proteins lost the ability to interact and inhibit
516 CPSF30 to improve viral fitness since this function is carried out by the viral PA-X
517 protein (64) and viruses encoding PA-X and NS1 proteins able to inhibit host gene
518 expression would not be as fitted as viruses where only one of the viral proteins is able
519 to inhibit host gene expression (65). It remains to be determined whether or not CIV
520 H3N2 PA-X does inhibit host gene expression to the same extend than H3N8 EIV
521 and/or CIV PA-X proteins.

522 In sum, our study provides insights into the mechanisms employed by IAVs to
523 counteract the innate immune response in different hosts and also how those can vary

524 depending on the virus origin and the strain involved. Future *in vivo* studies using these
525 viruses will shed light on the role of NS1 protein in pathogenicity, transmission efficiency
526 and mammalian adaptation in natural hosts.
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FIGURES

Figure 1. H3N8 CIV NS1 protein does not inhibit general gene expression in human HEK293T or canine MDCK cells. HEK293T **(A-C)** and MDCK **(D-F)** cells (12-well plate format, $3x10^5$ cells/well, triplicates) were transiently co-transfected with 50 ng of pCAGGS expression plasmids encoding GFP and Gluc together with 2 µg of pCAGGS plasmids encoding the indicated NS1 proteins fused to an HA-tag, or empty (E) plasmid as control. At 24 hpt, cells were analyzed for GFP expression using a fluorescent microscope (**A and D**) and Gluc activity from TCS (**B and E**). NS1 protein expression levels (**C and F**) from total cell lysates were determined by Western blot using an anti-HA polyclonal antibody (HA-NS1). Actin was included as a loading control. Representative images of three independent transfections are shown. Scale bar = 100 µm. Results represent the means and standard deviation of triplicate values. *, $p<0.005$ using Student's t test. PR8: influenza A/Puerto Rico/8/34 H1N1; BM/18: A/Brevig Mission/01/1918 H1N1; CIV NY09: A/canine/NY/dog23/2009 H3N8.

Figure 2. A single amino acid substitution (K186E) restores H3N8 CIV NS1's ability to block host gene expression. A) Amino acid sequence alignment of IAV NS1 proteins: Residues 70 to 194 from NS1 proteins that do not inhibit (PR8 and pH1N1) or inhibit (BM/18 and TX/91) host gene expression were aligned to H3N8 CIV NY09 NS1 protein (bottom). Gray shadow highlights amino acid changes in the H3N8 CIV NY09 NS1 effector domain, which could have a role in the lack of inhibition of host gene expression (P77, D96, T112, E139, F185, K186, K193 and I194). Abbreviations are as those described in Figure 1 legend. pH1H1: A/California/04/09 H1N1; TX/91: A/Texas/36/91 H1N1. **B-D**) **Inhibition of host gene expression:** MDCK cells (12-well

590 plate format, 3x10^5 cells/well, triplicates) were transiently co-transfected with pCAGGS
591 plasmids encoding GFP and Gluc (50 ng) together with plasmids encoding the indicated
592 HA-tagged H3N8 CIV WT or single amino acid mutant (2 µg) NS1s, or empty (E)
593 plasmid as control. At 24 hpt, cells were analyzed for GFP (**B**) and Gluc (**C**) expression.
594 NS1 protein expression levels from total cell lysates were determined by Western blot
595 using an anti-HA polyclonal antibody (HA-NS1) (**D**). Actin was included as a loading
596 control. Representative images of three independent transfections are shown. Scale bar
597 = 100 µm. Gluc expression levels in cells transfected with H3N8 CIV NS1 WT protein
598 were considered 100% for comparison to expression levels from H3N8 CIV NS1 single
599 amino acid individual mutant transfected cells. Results represent the means and
600 standard deviation of triplicate values. *, $p<0.05$ using Student's t test.

601 **Figure 3. Characterization of H3N8 CIV NS1 K186E mutation. A-B) Inhibition of**
602 **host gene expression:** MDCK cells (12-well plate format, 3x10^5 cells/well, triplicates)
603 were transiently co-transfected as described in Figure 1, using different amounts of
604 plasmids encoding N-terminal HA-tagged H3N8 CIV NS1 WT or K186E proteins. The
605 total amount of transfected plasmid DNA was maintained constant with empty plasmid.
606 At 24 hpt, cells were analyzed for Gluc activity from TCS (**A**). NS1 protein expression
607 levels were determined by Western blot using an anti-HA polyclonal antibody (HA-NS1)
608 and actin as a loading control (**B**). **C-E) H3N8 CIV NS1 K186E does not inhibit T7-**
609 **driven RNA polymerase expression**: Rodent BSRT7 cells (12-well plate format, 3x10^5
610 cells/well, triplicates) constitutively expressing the T7 RNA polymerase were transiently
611 co-transfected with the indicated NS1 expression plasmids or empty (E) plasmid as
612 control (2 µg), together with GFP and FFluc T7-driven reporter pCITE-T7 plasmids (0.5

613 µg). At 24 hpt, GFP expression was evaluated under a fluorescent microscope (**C**) and
614 Gluc activity was assessed from TCS (**D**). NS1 protein expression levels from total cell
615 lysates were determined by Western blot using an anti-HA polyclonal antibody (HA-
616 NS1) (**E**). Actin was included as a loading control. Representative images of three
617 independent transfections are shown. Scale bar = 100 µm. Results represent the means
618 and standard deviation of triplicate values. *, $p<0.05$ using Student's t test.
619 Abbreviations are as those described in Figure 1 legend.

620 **Figure 4. K186E substitution in H3N8 CIV NS1 protein restores binding to**
621 **CPSF30: A) Analysis of NS1-CPSF30 interaction by co-immunoprecipitation:** A
622 FLAG-tagged F2/F3 region of CPSF30 was expressed in HEK293T cells, mixed with *in*
623 *vitro* synthesized PR8 or H3N8 CIV (WT or K186E) NS1 proteins and
624 immunoprecipitated using an anti-FLAG resin. Following SDS-PAGE, input (top) and
625 immunoprecipitated (IP, bottom) proteins were detected by Western blot using
626 antibodies specific for the FLAG (region F2/F3 of CPSF30) or the HA (NS1s) tags. **B)**
627 **Tridimensional structure of NS1 effector domain in complex with the F2/F3 region**
628 **of CPSF30**: Influenza NS1 A/Udorn/72 H3N2 monomers conforming the dimer are
629 shown in magenta or blue and the monomers of F2/F3 region of CPSF30 in green or
630 brown. Amino acid residue K186 in influenza NS1 is indicated in yellow. The structure
631 was generated with Cn3D and it is based on the NS1 of influenza A/Udorn/72 H3N2
632 (PDB entry: 2RHK) (29).

633 **Figure 5. H3N8 CIV NS1 protein is an IFN antagonist:** HEK293T cells (12-well plate
634 format, $3x10^5$ cells/well, triplicates) were transiently co-transfected, using $CaPO_4$, with
635 reporter plasmids encoding GFP and FFluc under the control of the IFN-β promoter

636 (pIFN-β-GFP/CAT and p IFN-β-FFluc, respectively) (0.5 µg) (**A-C**) or expressing RFP
637 and FFluc under the control of an ISRE promoter (pISRE-RFP/CAT and pISRE-FFluc,
638 respectively) (**D-F**), and the indicated N-terminal HA-tagged NS1 protein expressing
639 plasmids, or empty (E) plasmid as control (1 µg). At 18 hpt, cells were infected (MOI 3)
640 with SeV, Cantell strain, and 18 hpi, cells were analyzed for IFN-β (**A-B**) or ISRE (**D-E**)
641 promoter activation by GFP (**A**) and RFP (**D**) expression under a fluorescent
642 microscope, and FFluc activity (**B and E**). NS1 protein expression levels from total cell
643 lysates were determined by Western blot using an anti-HA polyclonal antibody (HA-
644 NS1) **(C and F**). Actin was included as a loading control. Representative images of
645 three independent transfections are shown. Scale bar = 100 µm. FFluc activity is
646 represented as fold induction normalized to empty plasmid transfected, mock-infected
647 cells. Results represent the means and standard deviation of triplicate values. *, $p<0.05$
648 using Student's t test. Abbreviations are as those described in Figure 1 legend.

649 **Figure 6. Characterization of H3N8 NS1 WT and K186E CIVs. A**) **Phenotypic**
650 **characterization:** MDCK cells (6-well plate format, $1x10^6$ cells/well) were infected (MOI
651 of 3) with H3N8 NS1 WT and K186E CIVs. At 18 hpi RNA was collected and the NS
652 viral segments were amplified by RT-PCR and undigested (-) or digested (+) with SwaI
653 restriction endonuclease. DNA expected molecular sizes (nt) are indicated on the left.
654 **B**) **Plaque assay:** Plaque sizes of H3N8 NS1 WT and K186E CIVs in MDCK cells (6-
655 well plate format, $1x10^6$ cells/well) were evaluated at 3 days pi by immunostaining using
656 an anti-NP monoclonal antibody (HB-65). **C to F**) **Growth kinetics:** MDCK (**C and D**)
657 or A72 (**E and F**) cells (12-well plate format, $5x10^5$ cells/well, triplicates) were infected
658 with low (**C and E**) or high (**D and F**) MOI (0.001 or 3, respectively) and TCS were

collected at the indicated hpi. Viral titers were determined by immunofocus assay (FFU/ml). Data represent the means of the results determined for triplicate wells. *, $p<0.05$ using Student's t test.

Figure 7. Induction of innate immune responses by H3N8 NS1 WT and K186E mutant CIVs: MDCK cells (12-well plate format, $5x10^5$ cells/well, triplicates) were infected (MOI of 3) with H3N8 NS1 WT and mutant K186E CIVs. At 12 (**A and C**) or 24 (**B and D**) hpi total RNA was collected and mRNA expression levels of the ISG IFIT2 (**A and B**) and IFN β (**C and D**) were quantified by qRT-PCR analysis. Expression fold changes were calculated relative to mock-infected cells Data represent the average and standard deviation of triplicate values. *, $p<0.05$ using Student's t test.

Figure 8. The ability to block host gene expression was lost along the evolution of H3N8 EIV. A) **Amino acid sequence alignment of H3N8 CIV and EIV NS1 proteins**: Amino acid residues 161 to 230 from CIV NY09, A/canine/Florida/2004 (CIV FL04), A/equine/Pennsylvania/1/2007 (EIV Pen07) and A/equine/Miami/1963 (EIV Mi63) H3N8 NS1 proteins are shown. Gray shadow highlights amino acid 186. **B-D**) **Amino acid 186 is responsible for the lack of inhibition of host gene expression in H3N8 CIV and EIV NS1 proteins**: MDCK cells (12-well plate format, $3x10^5$ cells/well, triplicates) were transiently co-transfected with expression plasmids encoding GFP and Gluc (50 ng), together with plasmids encoding the indicated H3N8 CIV or EIV WT and mutant NS1 proteins, or empty (E) plasmid as control (2 µg). At 24 hpt, cells were evaluated for GFP expression (**B**) or Gluc activity (**C**). NS1 protein expression levels from total cell lysates were determined by Western blot using an anti-HA polyclonal antibody (HA-NS1) (**D**). Actin was included as a loading control. Scale bar = 100 µm. Results

represent the means and standard deviation of triplicate values. *, p<0.05 using Student's t test.

Figure 9. Frequency of identified NS1 186 mutation in H3N8 EIVs and CIVs isolates over time: Publicly available sequences in the Influenza Research database (www.fludb.org) were downloaded and the frequency of H3N8 EIV and CIV NS1 sequences containing amino acids E186 or K186 are represented according to the years of virus isolation.

Figure 10. The avian-origin H3N2 CIV NS1 protein is able to efficiently block host gene expression: MDCK cells (12-well plate format, $3x10^5$ cells/well, triplicates) were transiently co-transfected with expression plasmids encoding GFP and Gluc (50 ng) together with plasmids encoding the indicated NH2 HA-tagged H3N8 or H3N2 CIV NS1 proteins, or empty (E) plasmid as control (2 µg). At 24 hpt, cells were analyzed for GFP (**A**) and Gluc (**B**) expression. NS1 protein expression levels from total cell lysates were determined by Western blot using an anti-HA polyclonal antibody (HA-NS1) and actin was included as a loading control (**C**). Scale bar = 100 µm. Results represent the means and standard deviation of triplicate values. *, p<0.05 using Student's t test. H3N8 CIV: A/canine/NY/dog23/2009 H3N8; H3N2 CIV: A/canine/IL/41915/2015 H3N2.

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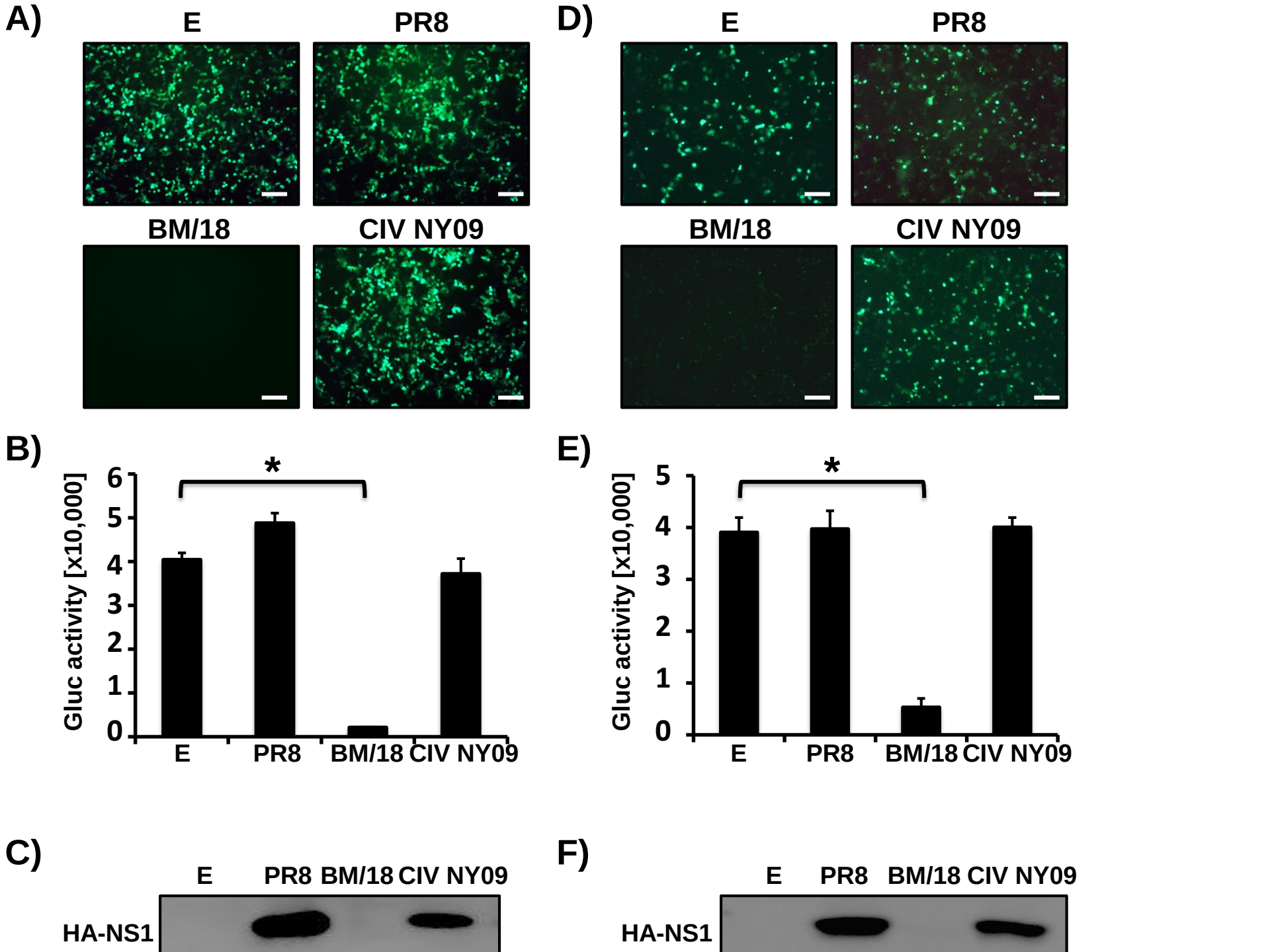
A)
E
PR8
BM/18
CIV NY09
D)
E
PR8
BM/18
CIV NY09
B)
Gluc activity [x10,000]
0
1
2
3
4
5
6
*
E
PR8
BM/18
CIV NY09
E)
Gluc activity [x10,000]
0
1
2
3
4
5
*
E
PR8
BM/18
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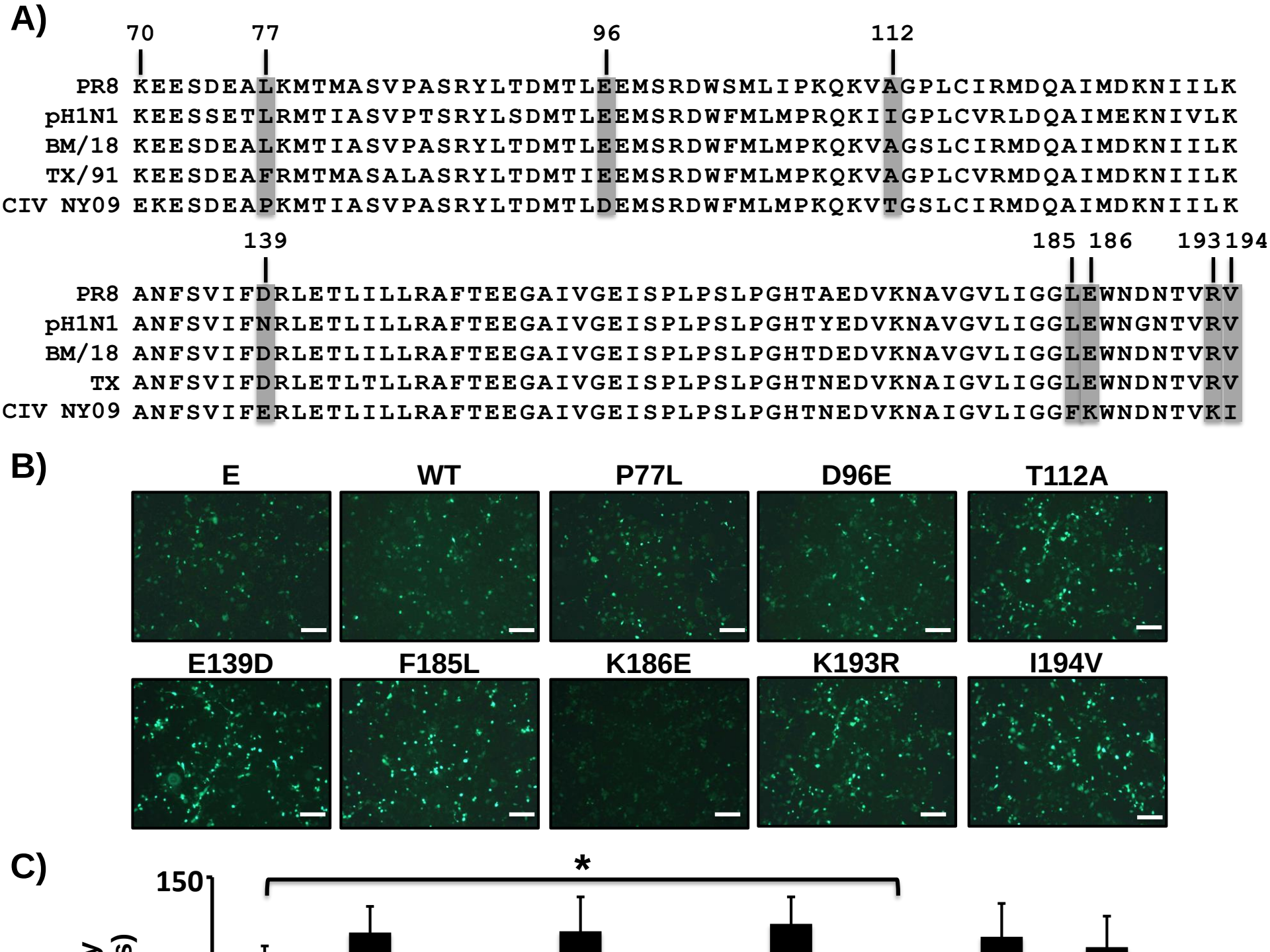
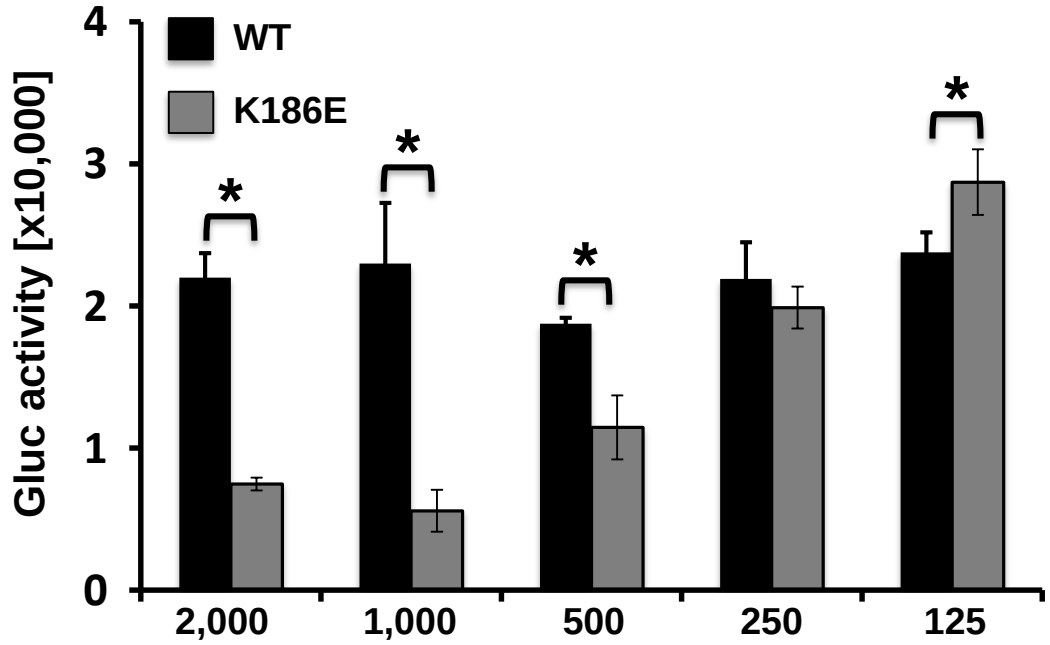
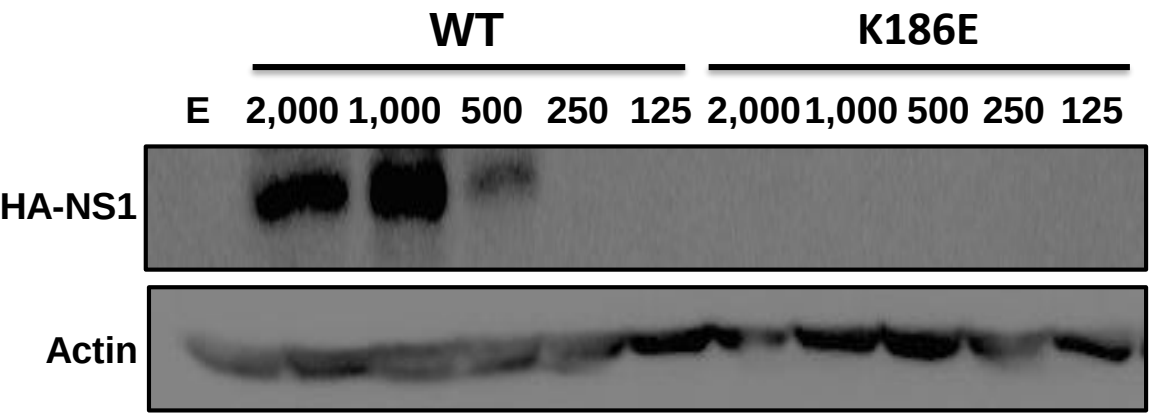
F)
E
PR8
BM/18
CIV NY09
HA-NS1


A)
70
77
96
112
PR8 KEESDEALKMTMASVPASRYLTDMTLEEMSRDWSMLIPKQKVAGPLCIRMDQAIMDKNIILK
pH1N1 KEESSETLRMTIASVPTSRYLSDMTLEEMSRDWFMLMPRQKIIGPLCVRLDQAIMEKNIVLK
BM/18 KEESDEALKMTIASVPASRYLTDMTLEEMSRDWFMLMPKQKVAGSLCIRMDQAIMDKNIILK
TX/91 KEESDEAFRMTMASALASRYLTDMTIEEMSRDWFMLMPKQKVAGPLCVRMDQAIMDKNIILK
CIV NY09 EKESDEAPKMTIASVPASRYLTDMTLDEMSRDWFMLMPKQKVTGSLCIRMDQAIMDKNIILK
139
185 186
193 194
PR8 ANFSVIFDRLETLILLRAFTEEGAIVGEISPLPSLPGHTAEDVKNAVGVLIGGLEWNDNTVRV
pH1N1 ANFSVIFNRLETLILLRAFTEEGAIVGEISPLPSLPGHTYEDVKNAVGVLIGGLEWNGNTVRV
BM/18 ANFSVIFDRLETLILLRAFTEEGAIVGEISPLPSLPGHTDEDVKNAVGVLIGGLEWNDNTVRV
TX ANFSVIFDRLETLTLLRAFTEEGAIVGEISPLPSLPGHTNEDVKNAIGVLIGGLEWNDNTVRV
CIV NY09 ANFSVIFERLETLILLRAFTEEGAIVGEISPLPSLPGHTNEDVKNAIGVLIGGFKWNDNTVKI
B)
E
WT
P77L
D96E
T112A
E139D
F185L
K186E
K193R
I194V
C)
*
150

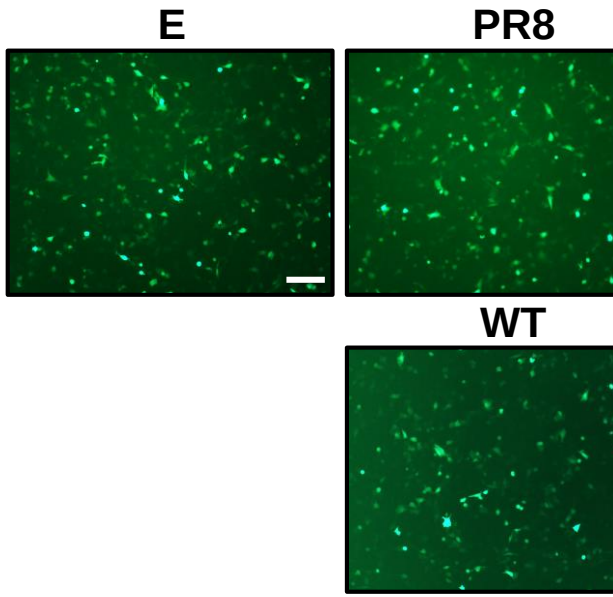
A)


WT
K186E
Gluc activity [x10,000]
4
3
2
1
0
*
*
*
*
2,000
1,000
500
250
125

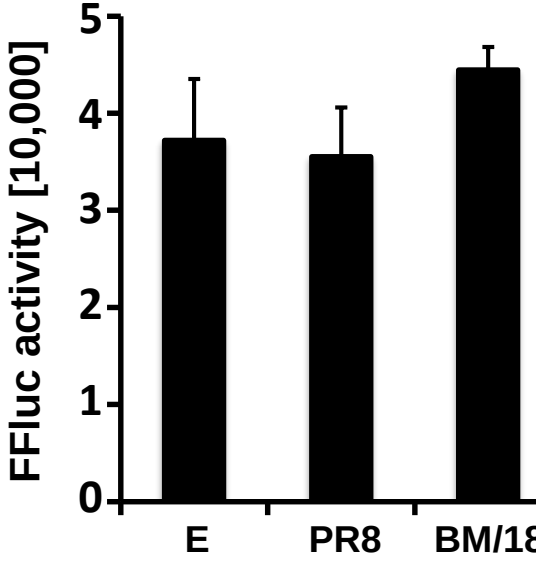
B)


WT
K186E
E
2,000
1,000
500
250
125
2,000
1,000
500
250
125
HA-NS1
Actin

C)


E
PR8
WT

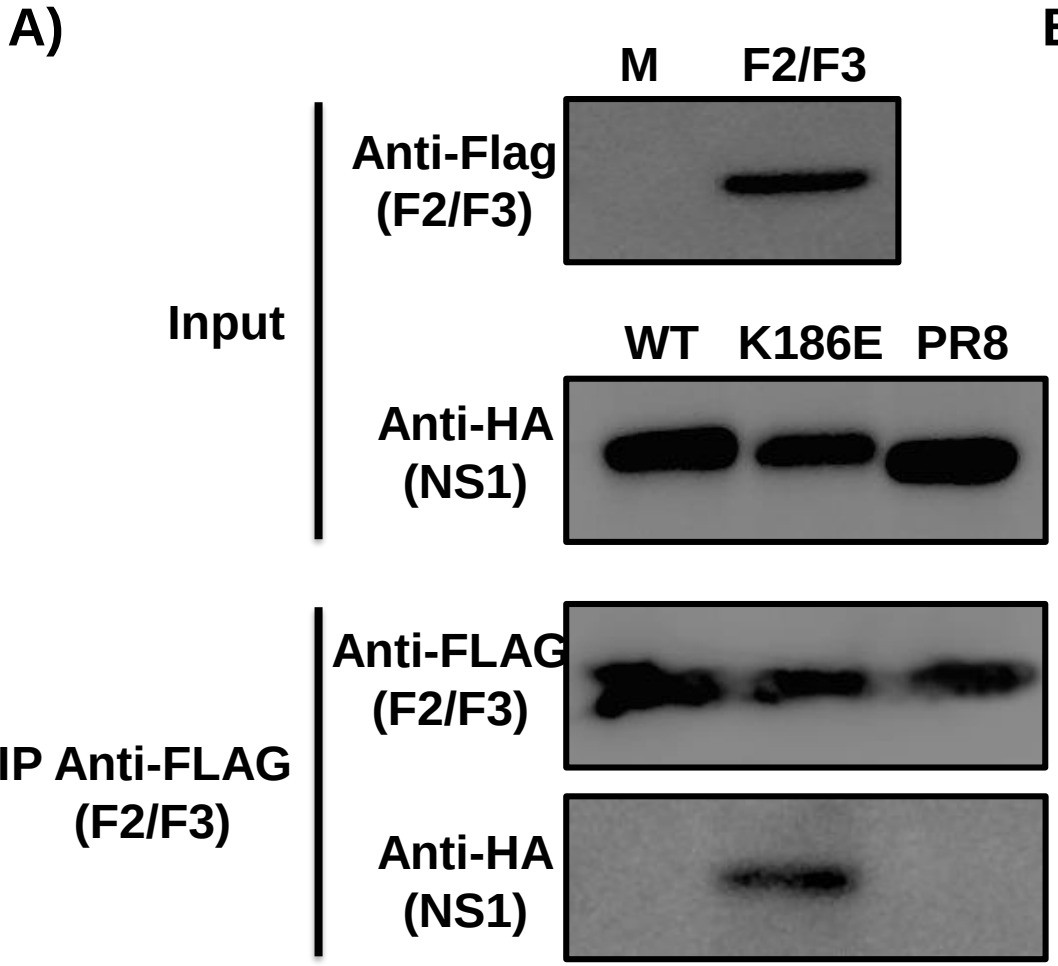
D)


FFluc activity [10,000]
5
4
3
2
1
0
E
PR8
BM/18

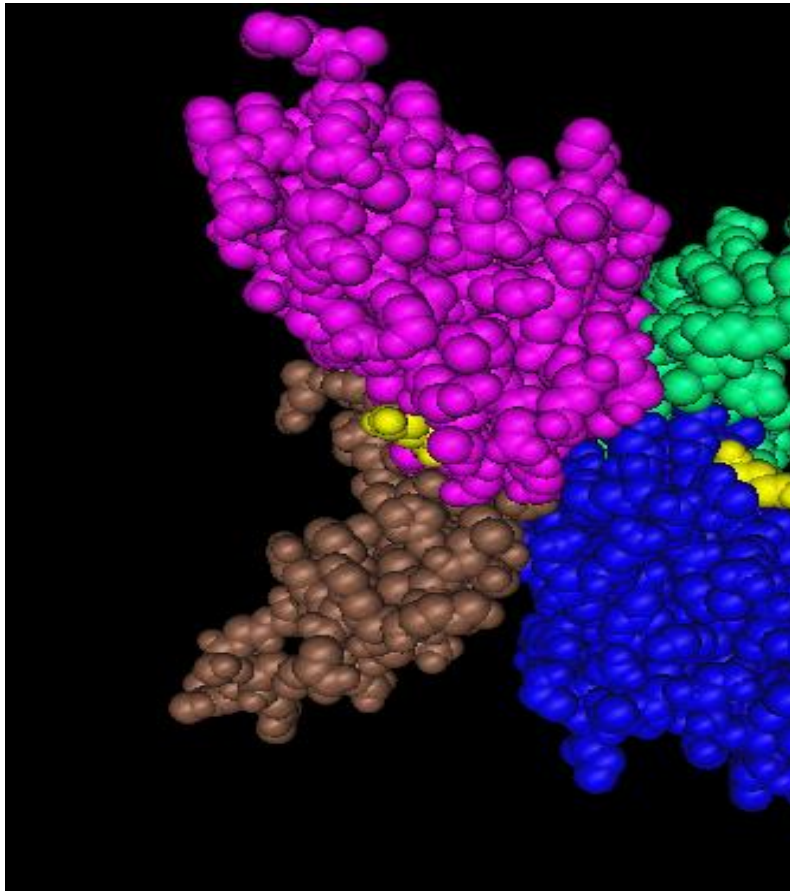
E)

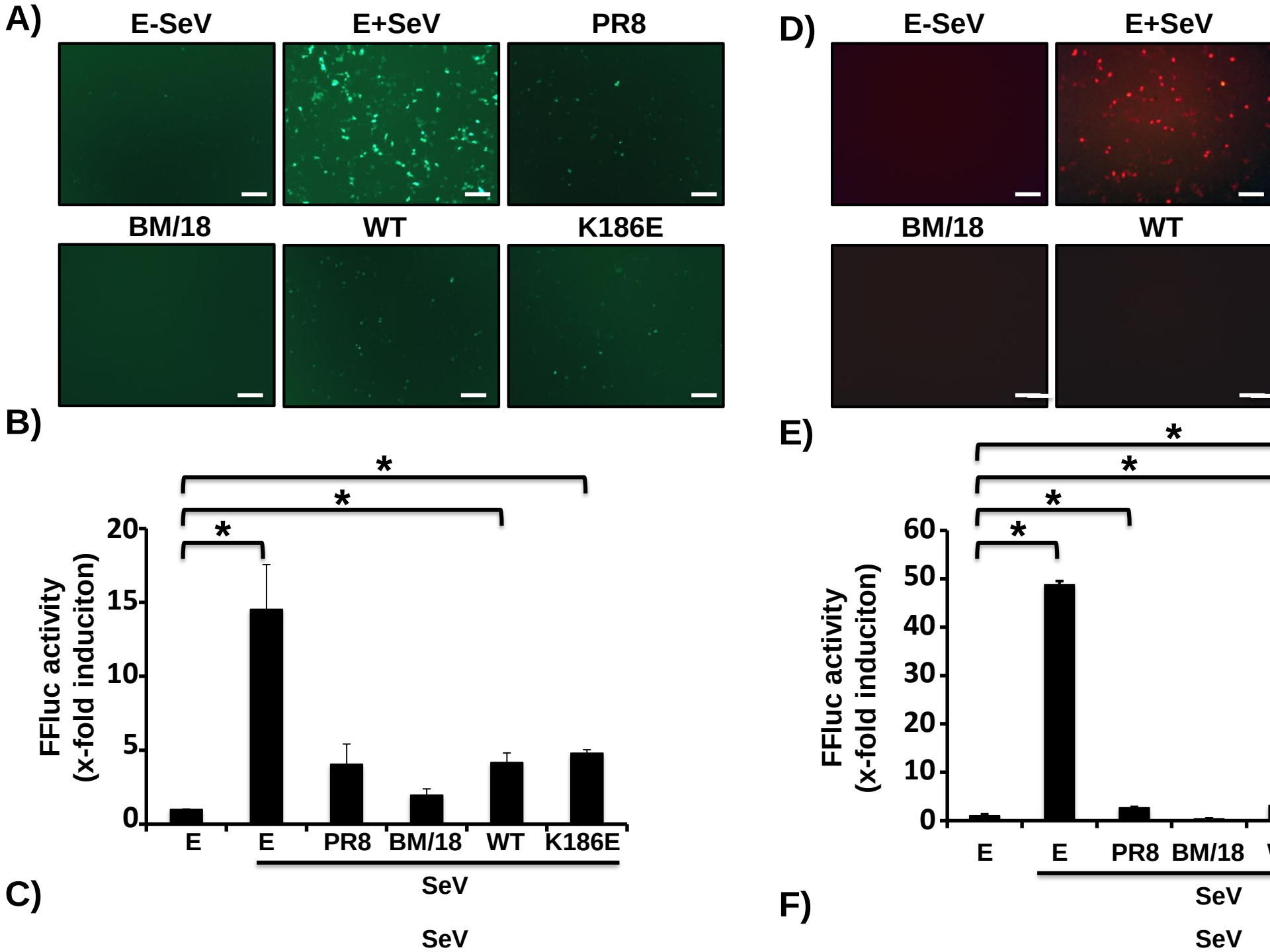

E
PR8
BM/18
HA-NS1

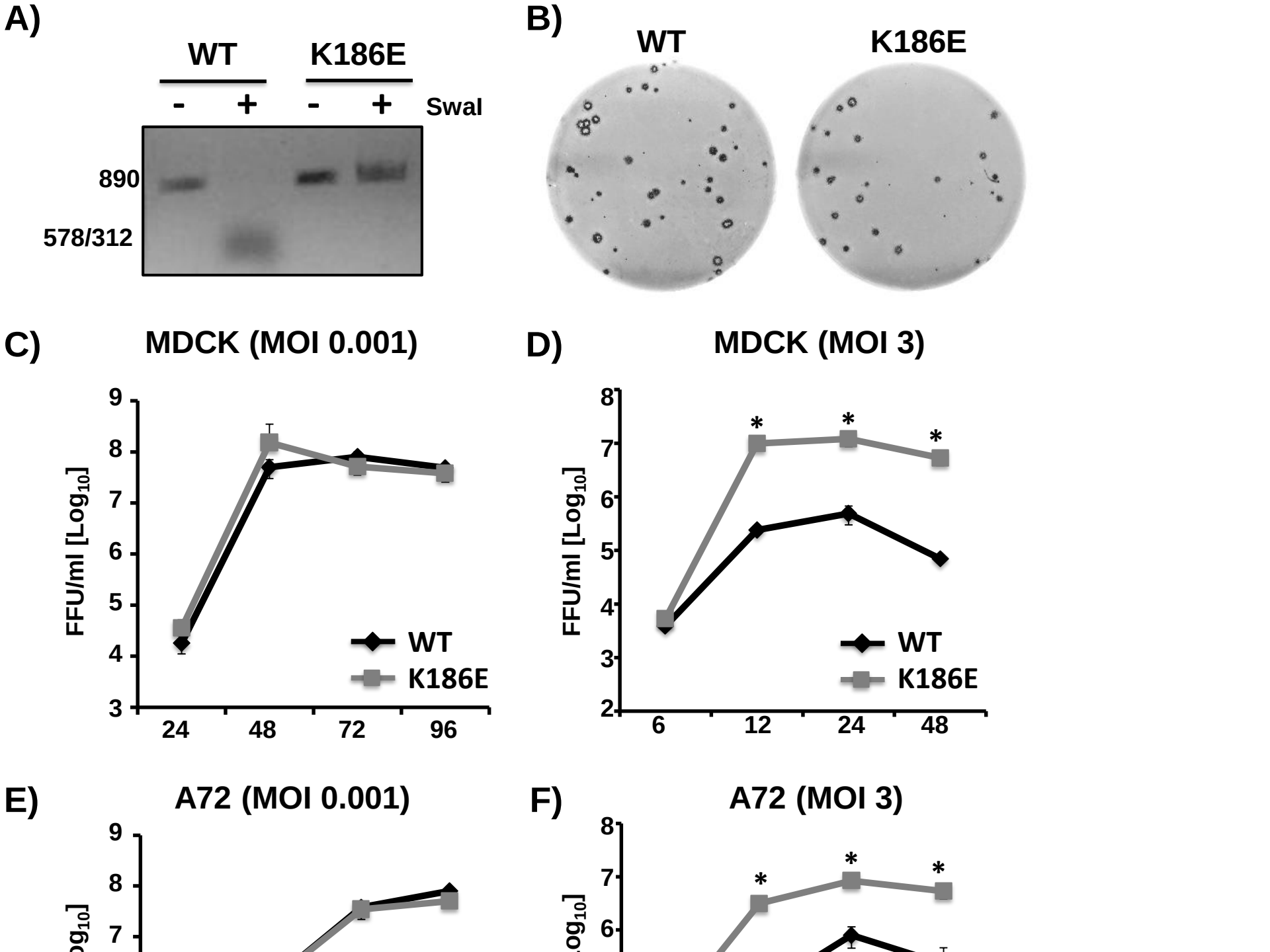
A)

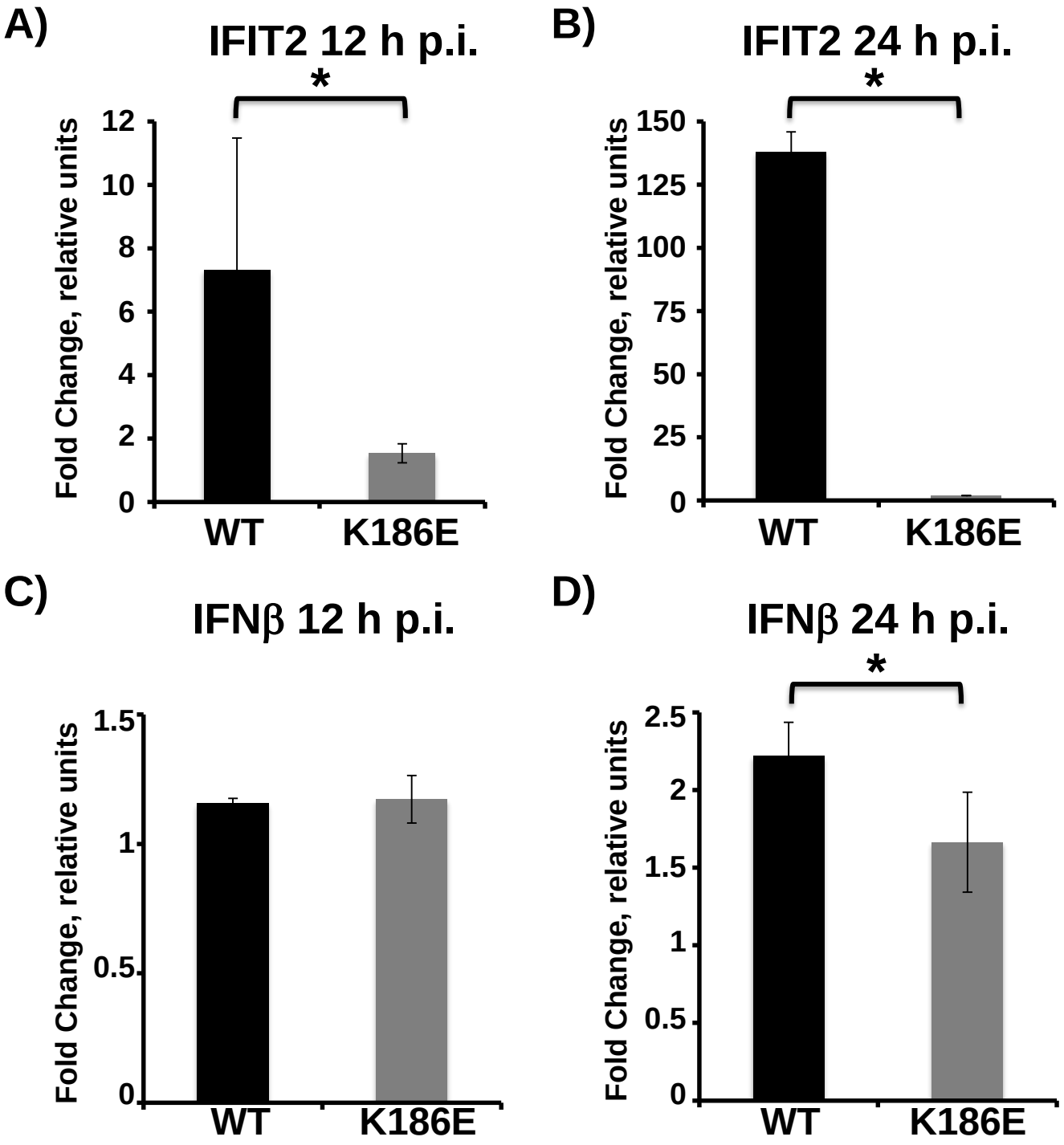

M
F2/F3
Anti-Flag
(F2/F3)
Input
WT
K186E
PR8
Anti-HA
(NS1)
Anti-FLAG
(F2/F3)
IP Anti-FLAG
(F2/F3)
Anti-HA
(NS1)

B)




A)
E-SeV
E+SeV
PR8
BM/18
WT
K186E
B)
FFluc activity
(x-fold induciton)
20
15
10
5
0
*
*
*
E
E
PR8
BM/18
WT
K186E
SeV
C)
SeV
D)
E-SeV
E+SeV
BM/18
WT
E)
FFluc activity
(x-fold induciton)
60
50
40
30
20
10
0
*
*
*
*
E
E
PR8
BM/18
SeV
F)
SeV


A)
WT
K186E
-
+
-
+
SwaI
890
578/312
B)
WT
K186E
C)
MDCK (MOI 0.001)
FFU/ml [Log10]
WT
K186E
24
48
72
96
D)
MDCK (MOI 3)
FFU/ml [Log10]
WT
K186E
6
12
24
48
E)
A72 (MOI 0.001)
F)
A72 (MOI 3)


A)
IFIT2 12 h p.i.
*
Fold Change, relative units
12
10
8
6
4
2
0
WT
K186E
B)
IFIT2 24 h p.i.
*
Fold Change, relative units
150
125
100
75
50
25
0
WT
K186E
C)
IFNβ 12 h p.i.
Fold Change, relative units
1.5
1
0.5
0
WT
K186E
D)
IFNβ 24 h p.i.
*
Fold Change, relative units
2.5
2
1.5
1
0.5
0
WT
K186E

A)

```
          161                                                                  219       230
CIV NY09  SPLPSLPGHTNEDVKNAIGVLIGGFKWNDNTVKISETLQRFAWRSSHENGRPSLPSKQKRKMERTIKPEI
CIV FL04  SPLPSLPGHTNEDVKNAIGVLIGGLKWNDNTVRISETLQRFAWRSSHENGRPSFPSKQK----------
EIV Pen07 SPLPSLPGHTNEDVKNAIGVLIGGLKWNDNTVRISETLQRFAWRSSHENGRPSFPSKQK----------
EIV Mi63  SPLPSLPGHTNEDVKNAIGVLIGGLEWNDNTVRVSETLQRFAWRSSHENGRPSFPPKQKRKMARTIESEV
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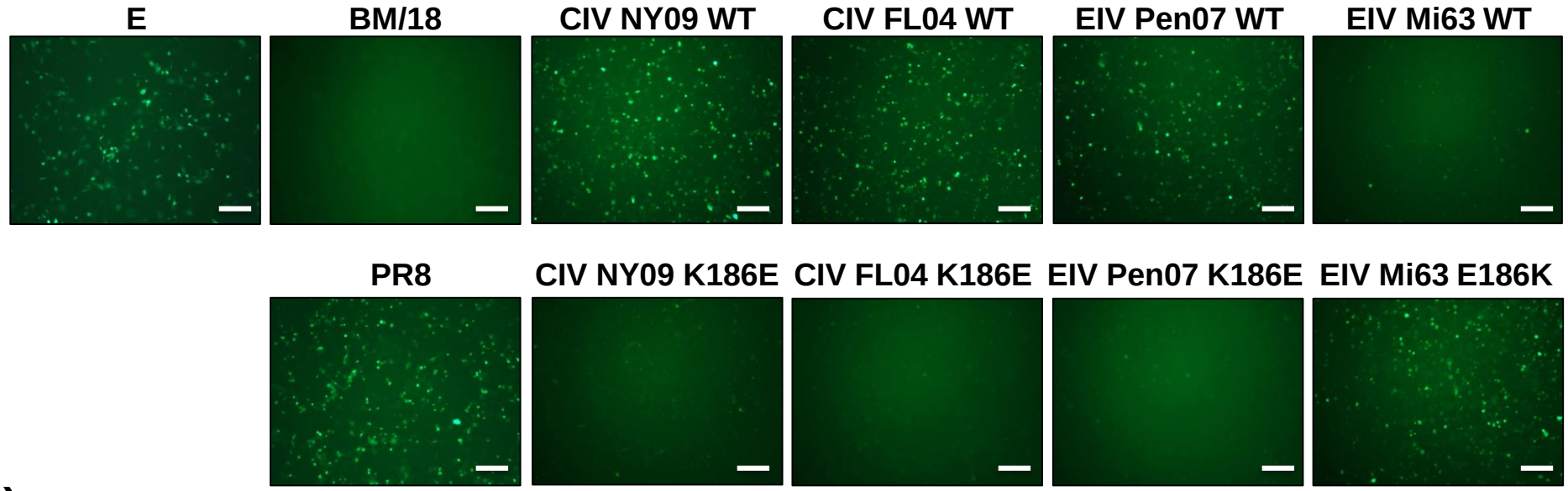
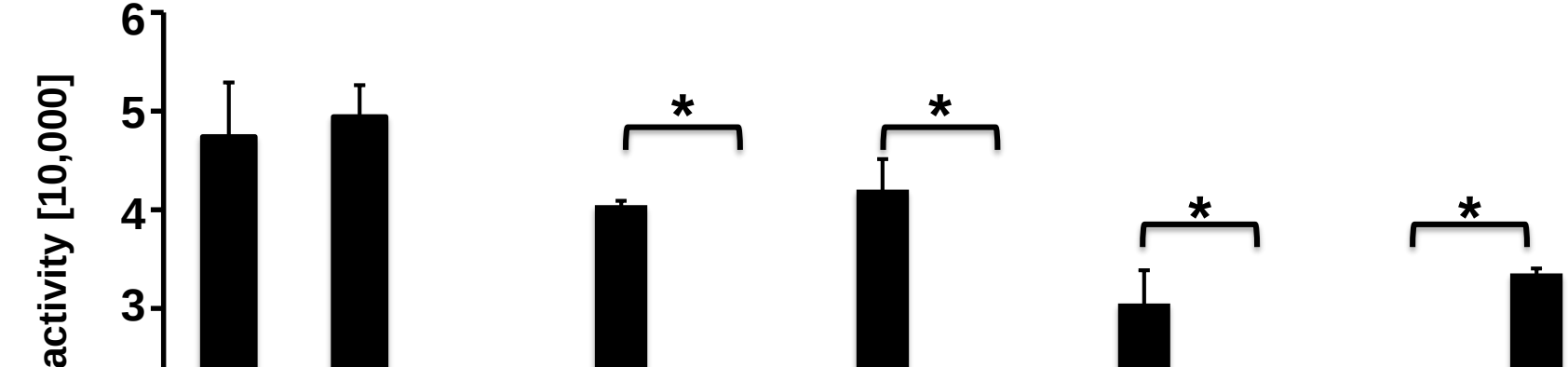
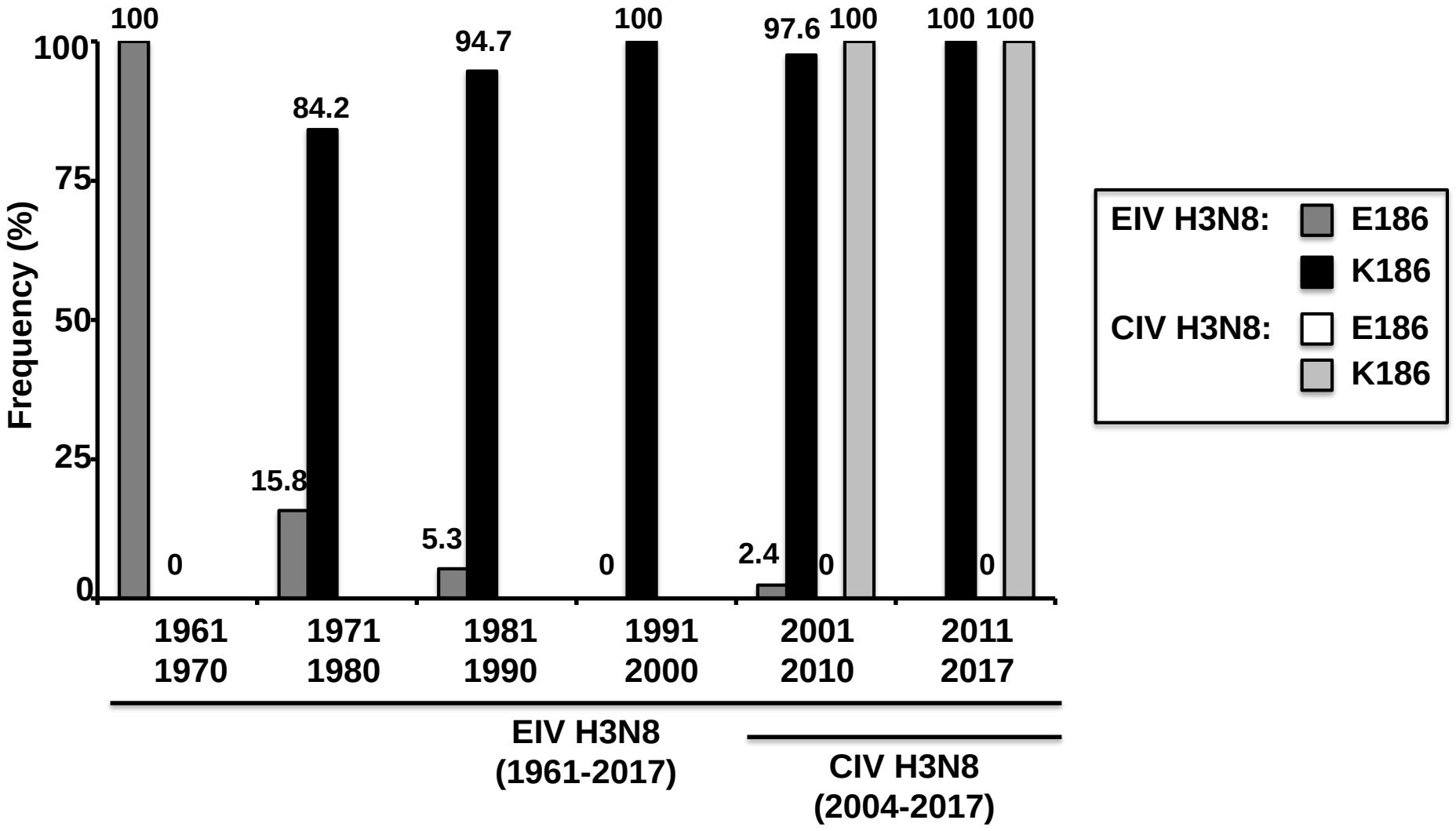
B)



C)

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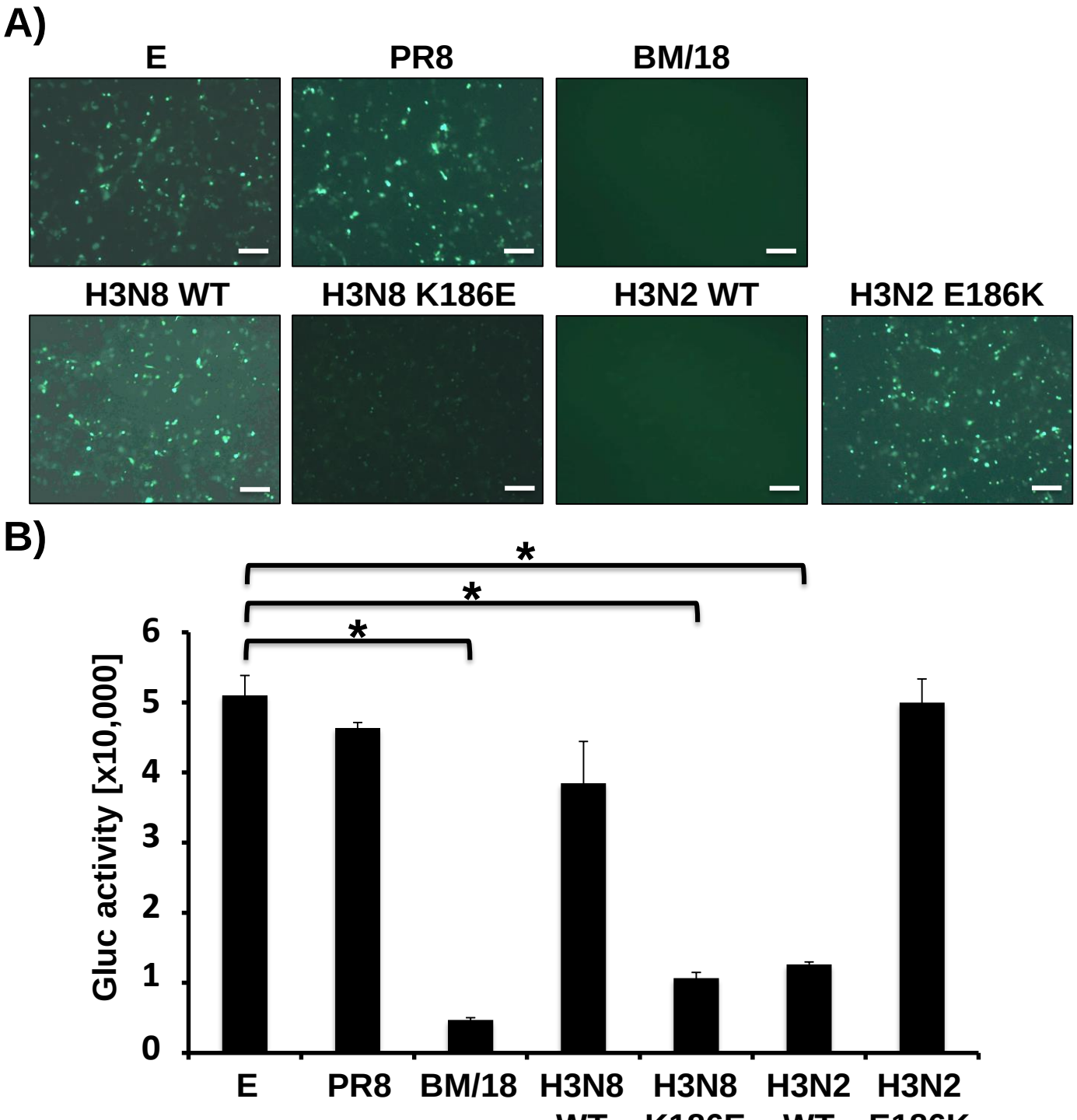
5.3
94.7
0
100
2.4
97.6
0
100
100
0
100
1961
1970
1971
1980
1981
1990
1991
2000
2001
2010
2011
2017
EIV H3N8
(1961-2017)
CIV H3N8
(2004-2017)
EIV H3N8:
E186
K186
CIV H3N8:
E186
K186


A)
E
PR8
BM/18
H3N8 WT
H3N8 K186E
H3N2 WT
H3N2 E186K
B)
*
*
*
Gluc activity [x10,000]
0
1
2
3
4
5
6
E
PR8
BM/18
H3N8
H3N8
H3N2
H3N2